

Free-Radical-Mediated DNA Binding

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Free-radical metabolites can be generated metabolically by a one-electron reductase-catalyzed reaction or a "peroxidase" catalyzed oxidation or by photoactivation of a wide variety of aromatic xenobiotics. Radicals may also be generated during lipid peroxidation. Some radicals can react with DNA or bind covalently or noncovalently as a dimerization product or as a dimer, trimer or polymeric product. Modification to the DNA can result in single-strand breaks, loss of template activity, and crosslinking. The binding can prevent enzymic digestion. In some cases, the radicals react with oxygen, resulting before conversion to DNA reactive oxygen species. Most radicals probably do not interact with DNA.

Introduction

As a result of the realization in the early 1940s that free radicals produced by ionizing radiation and ultraviolet radiation can lead to carcinogenesis, there has been interest in the possibility that free-radical metabolites of xenobiotics are possible toxic intermediates or proximate carcinogens. The most widely accepted theory for the initiation of processes leading to carcinogenesis is that carcinogens elicit their activity after conversion to electrophiles that interact with a nucleophilic region of a macromolecule. Binding to DNA in a way that causes errors in replication during cell division could result in initiation. Subsequent proliferation of these cells may lead to the neoplastic state. It would be expected that such binding should result in a minimal change in the physical structure of the DNA so as to avoid setting into motion the highly effective *in vivo* repair mechanisms. On the other hand, cytotoxicity could also occur if the binding to DNA prevents normal repair processes and the damage to the DNA results in an inhibition of RNA synthesis or DNA replication. In the case of the toxic and carcinogenic effects of irradiation, the importance of free-radical interactions with DNA have been reviewed previously (1).

The metabolic activation of chemical carcinogens in the liver is generally thought not to involve free radicals but rather electrophiles, e.g., nitrenium ions in the case of arylamines, carbonium ions in the case of polycyclic aromatic hydrocarbons. The first step in this activation is believed to involve a two-electron oxidation catalyzed by a cytochrome P-450-dependent mixed-function oxidase of the arylamine to the *N*-hydroxyaryllamine and the polycyclic aromatic hydrocarbon to an epoxide. Furthermore, the structure of the DNA adducts formed in the liver and some other target organs *in vivo* is identical to that formed *in vitro* with the proximate carcinogen formed by this pathway (2). However, one-electron oxidative or reductive activation of carcinogens involving free radicals and leading to extensive DNA binding has been proposed by various investigators for different carcinogens and certain target organs. The enzyme catalysts involved include peroxidases, prostaglandin synthetase and various reductases. In most nonhepatic tissues, these activities are often much more active than mixed function oxidase activity. Reviews on the prostaglandin synthetase catalyzed mechanism have appeared (3,4). There are also reviews available comparing one-electron and two-electron oxidative mechanisms for carcinogenic arylamines (5) and polycyclic aromatic hydrocarbons (6). Reductase catalyzed activation by the formation of organic and oxygen rad-

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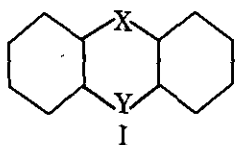
icals have also been reviewed (7,8).

Several investigators, however, believe that target tissues activate the detoxified conjugates released from the liver and transported in the blood or bile, e.g., by enzymic hydrolysis (9).

The present paper reviews various studies on DNA interactions with the free radicals of oxygen, anticancer drugs, mutagenic dyes, and a wide range of carcinogens. The reactive radicals can be generated from these compounds by reductive or oxidative metabolism or in some cases, by reductive or oxidative photoactivation. Oxygen radicals can also be generated by autooxidation of these radicals or other reduced metabolites. The nature of the DNA products formed and the effects on the DNA secondary and tertiary structure and consequences on DNA function are also discussed.

Noncovalent Binding

Many small positively charged molecules interact with nucleic acids. One general class of compounds which seem to intercalate between the bases in the double helix are those compounds with the structure I,



For acridines, $X = N$, $Y = C$; for phenazines, $X = N$, $Y = N$; phenothiazines, $X = N$, $Y = S$; actinomycin, $X = N$, $Y = O$. When base specificity is determined by differential dialysis of DNA bound by a series of proflavine and acridine orange analogs against DNA samples of differing G-C content, it is found that G-C specificity increases as the visible absorbance band of the chromophore moves to longer wavelength. This implies that as the G-C pair is more polar than A-T, it should interact more favorably with an easily polarized chromophore (10).

Other dyes used for staining chromosomes for which intercalation seems improbable as a result of their noncoplanar arrangement, include the common triphenylmethane dyes such as *p*-fuchsin, methyl green, crystal violet, and malachite green as well as the carcinogen auramine. All of these compounds show pronounced A-T specificity and bind to the outside of DNA in association with the negative phosphate groups (11). They can be removed from the DNA solutions at high salt concentration by two or three extractions with isopropanol.

Dye molecules of the aminoacridine class have frameshift mutational activity and the cationic form at a neutral pH form noncovalent complexes with DNA. At low concentration, the dye intercalates, but at high concentrations, binding to the outside of the double helix occurs (12). The intercalating dye can cause frameshift mutation in which a base is either inserted or deleted, resulting in a loss of register for the triplet code. The

N-methylphenazinium cation radical formed by a one-electron reduction of phenazine methosulfate binds to the DNA more strongly than the cation and the DNA stabilizes the cation radical against air oxidation and increases cation stability (13). The binding of both cations is reversed by raising the pH or ionic strength or by the presence of divalent cations. Binding of both cations also does not occur with single-stranded DNA (14). Proflavine binds equally well to double-stranded and single-stranded DNA indicating that ionic attraction of the phosphates for the cation dye is more important. However, NMR studies confirm the existence of π - π stacking between adenine bases and the *N*-methylphenazinium cation radical in the intercalation model (15).

Many antitumor drugs in clinical use may function as a result of their ability to bind noncovalently to DNA by intercalation (16). The anthracycline quinone, Adriamycin, is used to treat leukemias and solid tumors. Adriamycin and daunomycin intercalate DNA but favor G-C base pairs (17). This results in an increase of the DNA melting point. This stabilization of the DNA double helix could inhibit the formation of the single-stranded templates required by DNA and RNA polymerase and explains the inhibition of DNA replication and RNA transcription. Actinomycin D (containing a phenoxazone ring and peptide loop) intercalates and binds tightly to DNA at specific sequences (18) with a preference for G-C basepairs and causes very strong inhibition of polymerase action, particularly in some regions. By using a dry bound double-stranded plasmid pBR313 DNA and nick translation from a specific site, the sites at which bound drug molecules interfere with DNA polymerase action can be pinpointed. Ethidium bromide also seems to have a preference for intercalating G-C basepairs (17,19) but this is not a strict requirement. Distamycin A, with planar pyrrole rings connected by peptide bonds, preferably binds in A-T rich regions, probably in the minor groove (17). The inhibition of polymerase action may also be relevant to its antitumor action. The affinity of diacridines for DNA are greatly increased if the linker methylene chain is increased from four to eight carbon atoms, as both acridines can now intercalate between different pairs of adjacent DNA bases as measured by the removal and reversal of the supercoiling of covalently closed DNA. Much less binding to single-stranded DNA occurs (20).

The phenothiazine derivative, chlorpromazine, is widely used as a tranquilizer and an antipsychotic drug. The sulfoxide and the hydroxylated derivatives are the major *in vivo* metabolites and are also formed from the cation radical produced by a one-electron oxidation or by ultraviolet irradiation (21,22). The cation radical differs from chlorpromazine in being less folded and carrying a positive charge. The aromatic ring systems of phenothiazine cation radicals are flatter than phenothiazines as a result of less folding along the S-N axis of the ring system (23) and explains the formation of partial intercalation complexes with DNA. Evidence that intercalation, rather than external binding of DNA is

involved in the concomitant increased viscosity and the requirement for a double helix for stabilization of the cation radical (24). DNA shields the cation radical from reacting with peroxidase and nucleophilic buffer constituents. ESR spectral evidence of oriented DNA fibers from DNA solutions spin-labeled with the radical cation indicate that the DNA helix is perpendicular to the aromatic molecular plane of complexed cation radical (25). The radical is highly cytotoxic to *Salmonella* strains used for mutagenesis studies. However, evidence of repairable DNA damage was obtained for *E. coli* (24).

Bioreductive Activation

Mitomycin C is an anticancer drug that is selectively toxic to hypoxic tumor cells located in solid tumors that contain areas of vascular insufficiency. These oxygen-deficient cells are an obstacle to curative therapy, as the hypoxic stem cells are relatively resistant to X-irradiation. Mitomycin C is reduced by nuclear and microsomal NADPH-cytochrome P-450 reductase (27) to a semiquinone radical anion (26). Loss of methanol and a nucleophilic attack then results in covalent binding to DNA to form cis and trans monofunctional adducts. These adducts may then undergo secondary reduction and condensation reactions to yield bifunctional adducts which result in DNA crosslinking. The highly reactive quinone methides may be the alkylating species involved (28). Mitomycin C has also been shown to cause DNA strand scission *in vivo* and *in vitro*. Deoxyribose degradation readily occurs at even a low concentration of O_2 . Metal chelators markedly inhibited and hydroxyl radical scavengers partly inhibited. The responsible hydroxyl radicals were suggested to arise from a Fenton-type sequence in which the semiquinone radical reacts with H_2O_2 or reduces the ferric catalyst (29).

Although antitumor anthracycline quinone drugs could function by noncovalent binding to DNA (16), it is now widely accepted that radical-mediated DNA strand breaks are more important in accounting for cell death. The autoxidizable semiquinone-free radicals may be formed by microsomal or nuclear cytochrome P-450 reductase (30). Some DNA adduct formation with semiquinone radicals have been reported which was suggested to correlate with the ability of Adriamycin to induce sister chromatid exchanges (31). However, many fewer strand breaks occurred under nitrogen, indicating that activated oxygen species were involved.

Both anthracycline quinones (Adriamycin and daunomycin) have a high affinity for Fe^{3+} , and the complexes can undergo a self-reduction involving an intramolecular electron transfer, to form an iron II complex with the semiquinone radical (32). Superoxide radicals are then formed by autoxidation of Fe^{2+} , which can form H_2O_2 by dismutation and subsequently form $OH\cdot$ by a Fenton reaction. A species with a similar reactivity to hydroxyl radicals seem to be responsible for the subsequent degradation of deoxyribose and stimulation of lipid peroxidation (33). The Adriamycin- Fe^{3+} complex binds to DNA and catalyses its oxidative destruction.

Intercalated Adriamycin is not available for iron binding. In the presence of thiols and O_2 , the DNA cleaved (34). Furthermore, it has been found that the DNA-Adriamycin- Fe^{3+} ternary complex (unlike the corresponding bleomycin complex) is much more effective than the Adriamycin- Fe^{3+} complex in forming hydroxyl radicals from H_2O_2 (35). They also proposed that the iron chelation site of Adriamycin is between the hydroxyl at C_{11} and the carbonyl at C_{12} . One note of caution is recent evidence that membrane binding, rather than DNA changes, is responsible for the ability of Adriamycin to kill tumor cells (36). In this connection, the adriamycin-iron complex can bind to erythrocyte ghost membranes and in the presence of glutathione catalyzes the oxidative destruction of these membranes (37). *In vivo* lipid peroxidation has also been reported (38). Adriamycin also forms a strong electrostatic complex with cardiolipin, a phospholipid specific for this inner mitochondrial membrane. The subsequent membrane lipid peroxidation enables this complex to transport electrons and thereby interfere with normal mitochondrial electron transport (39).

Nitropyrene, nitrofurazone, and carcinogenic nitrofurans can be reductively activated to form nitrofuran-DNA adducts (40-42). However, in the case of the carcinogen *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide, the nitro anion free-radical product has been measured, but was found not to interact with DNA (43). This suggests that the DNA-reactive species is nitroso, nitroxide, or hydroxylamine formed by further reduction. DNA binding also occurred with the oxygen-insensitive *E. coli* reductase, even though no anion radical is formed. An oxidative activation to a different range of products with prostaglandin synthetase is also possible (44).

Adams and Dewey first suggested that other electron-affinic compounds could mimic the radiosensitizing effect of oxygen on hypoxic cells. Support for this suggestion is that these sensitizers have no radiation effect on aerated cells, and the ability to sensitize is closely correlated with the one-electron reduction potential, a measure of electron affinity determined by pulse radiolysis (45). Some nitroheterocycles, however, may be ineffective because metabolism reduces the effective drug concentration too rapidly. Various nitroheterocycles e.g., nitrofurazone, misonidazole, metronidazole (flagyl) have been shown to increase the sensitivity of hypoxic tumor cells to ionizing radiation. They are also useful for their selectivity as cytotoxic agents for hypoxic cells. The cytotoxicity seems to involve the depletion of GSH, membrane damage and DNA damage resulting in mutational activity. The latter may be important in explaining their carcinogenic activity. GSH depletion may be due to GSH adduct formation with nitroso compounds formed following reduction. Besides enhancing the effect of radiation on DNA, the radiosensitizers also act by removing GSH, thereby preventing repair processes from operating and thus further radiosensitizing hypoxic cells. Metronidazole, following reduction by microsomal reductases forms ac-

tive metabolites which bind to DNA and have mutational activity. The species involved may originate from hydroxylamino compounds formed following reduction (46).

Oxygen Radicals

In the interaction of hydroxyl radicals with DNA, 80% of the radicals add to the bases and less than 20% abstract an H atom from the sugar moiety (47). The formation of 8-OH-guanine (48) and thymine glycol (49) could prove to be useful for the determination of hydroxyl radical-mediated DNA damage *in vivo*. It has also been proposed that OH[•] adds to the double bond of the nucleic acid base to generate a radical which reacts with O₂ to form a peroxy radical. The latter reacts with the sugar base of another nucleotide and can result in a cleavage of the sugar. The sugar radical adds oxygen and the products hydrolyze, resulting in a strand break (50). Cleavage of the sugar also results in malondialdehyde formation (51) and has been used as a method for determining hydroxyl radicals in *in vitro* systems.

Exposure of DNA to superoxide-generating systems causes extensive strand breakage and deoxyribose degradation. Hydroxyl radical scavengers markedly protect (52). Similar results were obtained with rat liver nuclei incubated with Fe²⁺/O₂ and may be relevant to iron overload toxicity (53). Chromosome damage in mammalian cells also occurred (54).

The clinical effectiveness of bleomycins, a glycopeptide class of antibiotics, against Hodgkins lymphomas and squamous cell carcinomas and tumors of the testis, correlates with their capability to degrade DNA. Fe²⁺ complexes of bleomycin and oxygen readily cleave pBR 322 DNA, preferentially at G-C (5'—3') and G-T (5'—3') sequences. This specificity may be due to the interaction between the bleomycin bithiazole terminal amine moiety and the DNA guanine base. The metal binding site is thought to be the imidazole group. For effective DNA strand scission, the drug must deliver a metal ion to the site of the DNA helix attacked by the activated oxygen (55). Bleomycin can also preferentially degrade the DNA sequences in open chromatin within isolated nuclei (56). Hydroxyl radical formation has been demonstrated with certain spin traps (57) but it is not clear whether this radical is responsible for the strand scission. DNA strand scission seems to occur as a result of base propenal formation from an unstable 4-hydroperoxide formed by a radical abstraction of the hydrogen from the deoxyribose C-4 position. Malondialdehyde is released in the process (58). The release of free bases, however, is not due to a radical attack and can result in strand scission upon alkaline treatment (59).

The formation of active oxygen products such as hydroxyl radicals from the reaction of the mitomycin C semiquinone radical and bleomycin with oxygen may be responsible for the life-threatening interstitial pulmonary fibrosis in their clinical use (60,61). Myelosuppression occurs with most anticancer drugs, but not with bleomycin. Whether hydroxyl radical-induced DNA

strand scission is responsible for the cytotoxicity towards lung cells remains to be determined (62). Similarly, hydroxyl radicals formed by the autoxidation of the semiquinone radical formed by the reductive activation of Adriamycin (doxorubicin) may be responsible for the cardiomyopathy side effect (63,64). Lipid peroxidation readily occurs and has been implicated in the toxicity (33,65). Only after depletion of the mitochondrial GSH supply does extensive lipid peroxidation and damage to the plasma membrane of the intact cell occur (66). The cardiac myocyte may be a target because of the very low levels of catalase, superoxide dismutase, and the unusually large number of mitochondria in the cell. Active oxygen species may also be responsible for the Adriamycin-induced DNA strand scission found in tumor cells (30,67).

Adriamycin and daunomycin also degrade deoxyribose when enzymically reduced. Traces of dioxygen and ferric salts were required for the formation of a species with reactivity similar to that of a hydroxyl radical. It is possible an activated oxygen ferrous complex is responsible (68,69). Lipid peroxidation also occurs (33) when mitomycin C is enzymically reduced. However, effective anticancer drugs can act against well oxygenated as well as hypoxic cells in a gland tumor. Surprisingly though, activated oxygen is still formed under hypoxic conditions (29).

Toxic side effects are also a problem restricting the clinical use of the radiosensitizer misonidazole in the treatment of cancer. These include the development of a peripheral neuropathy and central nervous system damage. Nitrofurantoin treatment also results in pulmonary edema and fibrosis. This toxicity appears to be associated with an oxygen-sensitive reductive metabolism of the drug. The oxygen-sensitive nitroreductases reduce these radiosensitizers to nitro-anion free radicals which are oxidized by O₂ back to the radiosensitizer. In the process, superoxide radicals and H₂O₂ are formed and would also help to deplete GSH in the cell. DNA single-strand breaks observed with misonidazole in cultured mammalian cells (70) may be due to hydroxyl radical formation or radicals formed from nitro-anion free radicals.

Exposure of isolated rat hepatocytes to 2-methyl-1,4-naphthoquinone results in intracellular production of superoxide, oxidation of glutathione, plasma membrane bleb formation and loss of cell viability (71). A 27% increase in the fraction of single-stranded DNA was observed with 50 μM menadione. DNA binding, apparently by semiquinone, was also observed. Active oxygen species are believed to be responsible for the glutathione loss and DNA and membrane damage. The DNA strand breaks were actively repaired. Intracellular DT-diaphorase, which catalyzes the two electron reduction of menadione directly to the hydroquinone, protects the cells from the toxic effects by competing with the one-electron reduction pathways. Thus dicumarol, an inhibitor of DT diaphorase, markedly enhances cell toxicity (72).

Active oxygen, such as superoxide and H₂O₂, has re-

cently been shown to act as a promoter of transformation in mouse embryo fibroblasts (73) and induce chromosomal aberrations in human lymphocytes (74). Preneoplastic tissue is usually attacked by inflammatory leukocytes. Clastogenic factors are released by these leukocytes to induce chromosomal damage and interfere with normal intracellular metabolic cooperation associated with tumor promotion (75,76). The clastogenic factor may consist of free arachidonic acid and arachidonic acid hydroperoxides (77). Thus tumor promotion can be prevented by superoxide dismutase or biomimetic analogs (particularly with catalase), antioxidants or phospholipase inhibitors (73). These inhibitors also prevent the induction of ornithine decarboxylase in mice skin by a tumor promoter (75,78).

Oxidative Activation

Polycyclic Aromatic Hydrocarbons

A one-electron oxidation of polycyclic aromatic hydrocarbons (PAH) can result in cation radical formation. The covalent binding of PAH radical cations to nucleophiles depends on high localization at the most positive carbon atom in the radical cation. If the potential is below 7.35, the PAH binds to DNA, suggesting that only these PAH are activated by a one-electron oxidation (77). Peroxidase-hydrogen peroxide catalyzes the formation of a 6-(^{14}C)-methylbenzo(a)pyrene adduct with DNA. NMR analysis of the isolated adduct showed a covalent bond between the 6-methyl group of the 6-methylbenzo(a)pyrene and the 2-amino group of deoxyguanosine. A similar adduct coeluted with this adduct in a HPLC profile in an enzymic hydrolyzate of DNA isolated from the mouse skin 4 hr after a skin application of 6-(^{14}C)-methylbenzo(a)pyrene to mice. However, other adducts eluting before this adduct were not identified. The expected diol-epoxide adduct was not found (79). It was suggested that the radical cation reacts with nucleophilic groups of DNA by a prior loss of a methyl proton and an electron to generate a benzylic carbonium ion. The 6-methyl derivative was chosen because it specifically reacts with one-electron oxidants at the 6-methyl group. Benzo(a)pyrene binding to DNA also occurs during a peroxidase-catalyzed oxidation and the quinone products found are typical of a one-electron oxidation (80).

N-Oxidation

Hydrazine derivatives are widely used in medicine but often have tumorigenic and mutagenic properties. The nature of the lesions induced in the genetic apparatus is not known. A review on the biochemical toxicology of hydrazines is available (81). The monosubstituted hydrazines are readily oxidized by oxyhemoglobin, peroxidase, prostaglandin synthetase or cytochrome P-450 to carbon-centered radicals which can inactivate the heme catalyst (82,83). The tumorigenic hydrazine derivative 2-phenylethylhydrazine,

used as an antidepressant, is readily oxidized with oxyhemoglobin by a one-electron oxidation or microsomal mixed function oxidase mechanism. At the same time, strand breaks can be induced in supercoiled pBR 322 plasmid DNA, converting it to the open circular form. Spin trapping was used to show that the carbon radical yield correlated with the DNA nicking. Interestingly, oxygen was not required for the nicking, even though the inactivation of transforming DNA by other monosubstituted hydrazine derivatives has previously been ascribed to the active oxygen species formed (84). The metabolically generated carbon radical is therefore released from its site of formation and can induce DNA strand scission (81).

DNA binding readily occurred during the peroxidase- H_2O_2 catalyzed oxidation of the carcinogen aminofluorene, phenylenediamine and benzidine derivatives and methylaminoazobenzene (85). Radical cations are likely to be the first formed intermediates, so that it is of interest to investigate the role of these radicals in the binding. DNA was found to increase the stability of the radical cation for chlorpromazine, benzidine, or N,N',N,N' -(CH_3)₄-benzidine which, presumably, could reflect a more planar structure, able to intercalate DNA. The cation radical for durenediamine or phenylenediamine was also stabilized by DNA, although the cation radical for N,N -dimethylphenylenediamine was less stable (results not shown).

Aminofluorene is oxidized by a H_2O_2 -peroxidase reaction system to a purple intermediate product absorbing at 385 and 615 nm which is relatively stable at pH 5, but decays rapidly at pH 7.4. DNA stimulates the rate of formation of this intermediate, but inhibits its decay. At the same time, the intermediate forms a complex with the DNA with absorption maxima at 390 and 660 nm. The purple intermediate has been identified as 2-iminodifluorenylamine (86,87). Thus DNA enhances the rate of dimerization of aminofluorene cation radicals involved in 2,3-diaminofluorenylamine formation. Similar results were obtained with 2,3'-difluorenylamine, so that it is concluded that the monocation from the imine forms a complex with DNA. Most of the binding was non-covalent in nature and was removed by reduction and ethyl acetate extraction. The rest of the dimer and polymers could be removed with phenol treatment and protease treatment. However, with ^{14}C -aminofluorene, a small amount of radioactivity (approx. 0.5%) was covalently bound to the DNA.

At a neutral pH, the aminofluorene cation radical is probably uncharged, and N-N coupling resulting in azofluorene formation occurs. DNA seems to enhance the rate of dimerization by the cation radicals and by complexing the imine may hinder polymerization. The polymer is also complexed to the DNA. Thus an ethyl acetate extract shows much less dimer and polymer formation in the presence of DNA (86). It is unlikely that the DNA complexes with oxidation products involve intercalation, as single-stranded DNA is twice as effective as double-stranded DNA in binding the products (88). The aminofluorene DNA adduct seems to in-

volve the guanine base and to a lesser extent adenine as ^{14}C -aminofluorene binds to polyriboguanilyc acid much more than polyriboadenilyc acid. Little binding occurred to polyribouridylic acid and polyribocytidylic acid (88). DNA adduct analysis shows a small amount of C8-guanine adduct, presumably as a result of nitrosofluorene formation, but the major early eluting adduct is unidentified (89).

Both liver and mammary gland of the male and female rat, respectively, are the sites of carcinogenic action of *N*-hydroxy-*N*-2-fluorenylaceta-mide (90). A one-electron oxidation of this carcinogen to the nitroxyl free radical has been shown on incubating rat mammary gland cells with arachidonate (91). A similar oxidation occurs with peroxidase- H_2O_2 (92). As mammary gland has very low levels of cytochrome P-450 (93), it has been suggested that the active catalysts are lipoxigenase (91) or cytochrome c (93). The nitroxyl free radicals dismutate to equimolar amounts of nitrosofluorene and *N*-acetoxy-*N*-2-fluorenylaceta-mide, and the latter forms a covalent adduct with nucleic acids when a peroxidase- H_2O_2 system was used as the one-electron oxidizing system for forming the nitroxyl free radicals (92). The reactive species involved in the peroxidase- H_2O_2 -catalyzed DNA binding of *N*-hydroxy-*N*-2-fluorenylaceta-mide are different from that for aminofluorene (88).

Phenylenediamine derivatives are components of permanent and semipermanent hair dye formulations and synthetic rinses. The peroxidase- H_2O_2 system also readily oxidizes *p*-phenylenediamine and 2,4-diaminoanisole to cation radicals, which are further oxidized or disproportionate to form a very reactive diamagnetic benzoquinonediimine. The latter is then oxidized to trimers known as Brandowski's base. DNA binding accompanies this oxidation at pH 5 (5). Furthermore, DNA binding still occurred even if the DNA was added 5 min after the reaction was stopped. Hydrogen peroxide also greatly enhances the mutagenicity of several phenylenediamines and has been attributed to the trimer Brandowski's base (94). Brandowski's base was synthesized from *p*-phenylenediamine and was found to bind to DNA to form a purple complex only after peroxidase- H_2O_2 catalyzed oxidation. This suggests that the imine derivatives of these trimers bind to DNA (5). The DNA binding was resistant to extraction with organic solvents or phenol or protease incubation. However, enzymic digestion of the bound DNA to nucleotides shows that most of the binding was noncovalent in nature. Similar results were obtained with 2,4-diaminoanisole or *p*-aminophenol. Again, the DNA binding was due to complex formation with the imine derivative of the equivalent Brandowski's base (5).

The cation radical for *p*-phenylenediamine (as determined by ESR) can be stabilized by N-CH_3 or O-CH_3 substitution (95,96). Thus the radical cations of *N,N',N,N'*-(CH_3)₄-*p*-phenylenediamine (known as Wurster's Blue) and durenediamine are stable. The radical cations of the N-CH_3 substituted *p*-phenylenediamine exist as stable paramagnetic salts. Durenediamine and *N,N*-(CH_3)₂-*p*-phenylenediamine did not bind

to DNA after a peroxidase- H_2O_2 catalyzed activation. DNA also had no effect on the *N*-demethylation of *N,N*-(CH_3)₂-*p*-phenylenediamine as measured by HCHO formation or cation radical (Wurster's Red) formation. Cation radical decay, however, was enhanced by DNA. *N,N',N,N'*-(CH_3)₄-benzidine was much less effective than benzidine in binding to DNA after a peroxidase- H_2O_2 catalyzed activation. The binding was found to occur following *N*-demethylation. Phenol conjugate formation also occurred following *N*-demethylation to *N,N*-(CH_3)₂-benzidine (97).

Benzidine and benzidine congener-based dyes are widely used in dye manufacturing, textile dyeing, paper printing and leather industries (98). The incidence of urinary bladder cancer is also elevated among industrial workers exposed to benzidine-based dyes. Of concern to the public is that mutagens and monoacetyl benzidine appear in the urine of rats or humans treated with benzidine congener-based azodyes (99). DNA-bound benzidine metabolites were also found in the livers of rats (100). Anaerobic intestinal bacteria are probably largely responsible for the azoreduction of these dyes and but liver azoreductases could be involved in the liver carcinogenicity (101).

In a comparison of a wide range of various phenols and arylamines in a peroxidase- H_2O_2 reaction mixture, the most extensive DNA binding occurred with benzidine (85). Similar results were found in another system, i.e., prostaglandin synthetase-arachidonate (102). Bladder transitional epithelial microsomes from dog, on addition of arachidonate, also catalyzed the binding of benzidine or *o*-dianisidine to DNA (103). The nature of the oxidation products including the first formed cation radical in a peroxidase- H_2O_2 catalyzed oxidation has been reviewed (104). The effects of DNA (105) on this reaction sequence are as follows. DNA does not affect the conversion of benzidine to radical cation but slows the rate of formation of quinonediimine from benzidine cation radical. DNA forms a complex with the quinonediimine of 3,3',5,5'-(CH_3)₄-benzidine, 3,3'-(CH_3)₂-benzidine or 3,3'-(OCH_3)₂-benzidine. This DNA interaction results in a spectral shift of the absorbance maxima of the quinonediimine of 6-12 nm to a longer wavelength. No complex is formed with single-stranded DNA. This suggests that the quinonediimine is planar and intercalates the base pairs. An electrostatic interaction between the monoprotonated diimine and the negative charge on the sugar phosphate groups may also be involved.

In the case of benzidine and 3,3'-(CH_3)₂-benzidine, DNA catalyzes a rapid decay of synthesized quinonediimine. At pH 6.5, azo compounds and polymer are the products but are bound to the DNA and cannot be extracted with ethyl acetate (85). At pH 5, a stable charge transfer complex and polymer bind to the DNA. The DNA therefore accelerates the conversion of quinonediimine to azo and polymer compounds. With single-stranded DNA and polyribonucleotides, no bound charge-transfer complex was formed although quinonediimine decay was accelerated. This suggests that the

intercalated diimine can form a charge-transfer complex. The reaction mixture containing DNA forms a thick gel if the DNA is at a concentration of 1 mg/mL which could also explain the stability of the charge-transfer complex.

MgCl₂ could prevent the enhanced quinonediimine decay by DNA suggesting that the negative phosphate groups of the DNA were involved. Up to 97% of the benzidine binds to the DNA following a peroxidase-catalyzed oxidation. The bound benzidine is not removed by butanol extraction, by phenol treatment, or pronase digestion. However, acid digestion releases the bound benzidine as polymeric material. Most of the bound DNA resists enzymic digestion by DNAase, phosphodiesterase, or alkaline phosphatase. Of the nucleotide:benzidine adducts, 7% extract into *n*-butanol. Separation of the adducts on HPLC showed that the major adduct (82% of the radioactivity) had a UV spectra with an absorbance maxima at 295 nm and a shoulder at 265 nm. The adduct was also degraded by 70°C for 1 hr or on adjusting the pH to 1 resulting in its conversion to an adduct having a maximal absorbance at 255 nm (85). These properties were similar to those reported for the *N*-acetylbenzidine adduct formed in rat liver *in vivo* (105), suggesting that the adduct is *N*-(deoxyguanosin-8-yl)-benzidine.

Recently, a *N*-acetylcysteine conjugate has been isolated from a peroxidase-H₂O₂-benzidine-*N*-acetylcysteine reaction mixture and also identified as 3-(*N*-acetylcysteine-S-yl)-benzidine (106). An analogous GSH conjugate has also been isolated from a prostaglandin H synthase-arachidonate-benzidine-GSH reaction mixture (107). The DNA adducts isolated from the dog bladder after the *in vivo* ¹⁴C-benzidine administration was 10% *N*-(deoxyguanosin-8-yl)[*N'*-acetylbenzidine but the rest was unidentified. In dog liver, less than 1% of the adducts were identified as this adduct (108). Recently, the *N*-(deoxyguanosin-8-yl)-benzidine has been identified as the major adduct isolated from the dog bladder. A similar adduct was found when benzidine-diimine was reacted with DNA (109).

A comparison of the various benzidine derivatives show that all of them bind to the DNA except 3,3',5,5'-(CH₃)₄-benzidine (110,111). This suggests that an unsubstituted ortho position is required for binding. This derivative is also noncarcinogenic in short-term tests (112), and azo dyes have recently been prepared for this derivative with the hope of making noncarcinogenic azo dyes available (113).

The above results also suggest that the major part of the binding may involve very tightly bound benzidine "melanin" which prevents enzymic digestion. Benzidine bound polyriboguanilyc acid also resisted ribonuclease digestion. This suggests that such binding *in vivo* would not be repairable and should be highly cytotoxic.

When covalently closed circular plasmid DNA pBR 322 is reacted with the benzidine oxidation products and subjected to gel electrophoresis, the bound DNA did not migrate or enter the gel. This suggests extensive crosslinking as the reaction conditions did not involve gel formation, and intercalation seems unlikely, as the

DNA still stained with ethidium bromide. Crosslinking was suggested to involve the benzidine diimine acting as a bifunctional alkylating agent (85).

N,N-Dimethyl-4-aminoazobenzene (DAB) is a well known liver carcinogen in rats and induces bladder tumors in dogs. Nonextractable DNA binding occurs during the *N*-dealkylation catalyzed by a peroxidase-H₂O₂ system. Methyl-4-aminoazobenzene (MAB) bound more effectively. Bladder microsomes and arachidonate also catalyzed this DNA binding. This was prevented by the prostaglandin synthetase inhibitor indomethacin (114). Lipid peroxides in the presence of heme also catalyzed the *N*-dealkylation of DAB, and in the presence of GSH, the GSH conjugate GS-CH₂-aminoazobenzene was isolated (86). This conjugate was also isolated from a reaction mixture of peroxidase-H₂O₂-MAB. When DAB is given in uninduced rats, the GS-CH₂-aminoazobenzene forms the major biliary metabolites. Indeed GSH depletion *in vivo* markedly decreased the biliary excretion (115). It is believed that this GSH conjugate is formed during *N*-demethylation of MAB by cytochrome P-450-dependent mixed-function oxidase. It is interesting that the same conjugate may also be formed by one-electron oxidative systems involving free radicals.

The main products formed by the peroxidase catalyzed oxidation of MAB at a neutral pH were identified as dimers formed by N-N coupling whereas at pH 5, N-C coupling predominated. The DNA binding was nonextractable and up to 5% of the MAB could be bound. However, the binding was noncovalent and enzymic digestion was required to release the radioactivity. A spectral analysis of the MAB bound DNA showed that the binding species was a dimer or polymer.

Many carcinogenic or genotoxic arylamines, e.g., 4-aminobiphenyl, 2-aminofluorene and benzidine or phenylenediamine derivatives are *N*-acetylated by the liver in many species *in vivo*. Little *N*-acetylation, however, occurs in the dog. Furthermore, the target organ in rodent species is the liver but in the dog, it is the bladder. *N*-acetylated arylamine:DNA adducts are among the DNA adducts isolated from rodent liver after the *in vivo* administration of these carcinogenic arylamines. By contrast, the adducts isolated from rodent nonhepatic tissues or dog liver were not acetylated (5). This suggests that the *N*-acetylated adducts may play a role in liver cancer. We have shown that *N*-acetylation of these arylamines prevents their oxidation by various one-electron oxidizing systems and enhances their rate of *N*-hydroxylation by liver cytochrome P-450-dependent mixed-function oxidase. However, in the presence of liver microsomes or esterase, the *N*-acetylarylamines are readily oxidized by one-electron oxidizing systems and that paraoxon completely prevents this oxidation to DNA reactive species. Clearly microsomal deacetylase activity is involved in this activation (88,116). The acetylase activity versus the deacetylase activity of a tissue may therefore be as important as the "peroxidase" activity versus the mixed-function oxidase activity in deciding whether free radicals are involved in the activation of these arylamines by various target tissues. Thus, the Zymbal gland may be an important target

organ because of its high deacetylase activity and peroxidase activity (88).

Phenols

Diethylstilbestrol induces genital tract tumors in young women whose mothers were treated with this synthetic estrogen during gestation. It is also carcinogenic in rodents. The hormonal activity of this estrogen is not sufficient to explain its tumorigenicity (117,118). The uterus has an estrogen-inducible peroxidase and prostaglandin synthetase activity (but no mixed-function oxidase activity) and peroxidative metabolism results in DNA binding (119,120) and genotoxic effects not including bacterial mutagenicity (121). In a recent study of various analogs, it was concluded that only those able to form phenoxy radicals bound and that the stilbene moiety was not required (122). The phenoxy radical (diethylstilbestrol-4',4"-semiquinone) is an intermediate for the formation of 4',4"-quinone which in protic solvents is converted to dienestrol. This conversion also occurs *in vivo* (121). It has been shown that DNA catalyzes the conversion of quinone to dienestrol and that in the process, the quinone binds to calf thymus DNA. The binding decreases with increasing reaction time (123). The bound diethylstilbestrol cannot be removed, but no bound nucleotides were found on enzymic digestion which indicates that the binding is noncovalent (unpublished data).

A chronic exposure of benzene produces hemopoietic toxicity and in severe cases results in aplastic anemia. It has also been implicated as a leukemogen (124). Benzene requires metabolic activation to produce its toxicity and a metabolite of phenol, not benzene oxide, was responsible for the majority of *in vitro* covalent binding to microsomal protein (125). Recently, we have shown that a peroxidase found in bone marrow oxidizes ^{14}C -phenol to various dimers and polymers via phenoxy radicals and in the process, up to 50% of the radioactivity bound to DNA (126). No catechol or hydroquinone was formed. No DNA binding occurred with the microsomal mixed-function oxidase with phenol, hydroquinone or catechol although protein binding did occur. Phenol was metabolized to hydroquinone and catechol in this system (127). Binding to mitochondrial DNA in rabbit bone marrow incubated with ^{14}C -benzene has also been reported recently (128). The nature of the reactive species formed from phenoxy radicals that bound to DNA was found to be a polymer of 5 phenol residues formed in the reaction mixture from *o,o*-biphenoxy and phenoxy radicals. Most of the binding was non-extractable but noncovalent as enzymic digestion released the radioactivity (129).

Ellipticine has been found to be an effective drug against murine leukemia L210 and several solid tumors. The mechanism seems to involve a metabolic conversion to the 9-hydroxy derivative which can intercalate DNA or undergo a one-electron oxidative activation to autoxidisable quinoneimine radicals which can bind to or damage DNA or bind covalently to protein (130,131).

The anticancer drug etoposide (VP-16) has been shown to form a phenoxy radical on oxidation by a peroxidase- H_2O_2 -system. This radical has been implicated in the DNA-breakage and cytotoxicity (132).

Polyunsaturated Fatty Acids

Carcinogenesis induced by chlorinated hydrocarbons, hydrazines, hypolipidemic drugs or choline-methionine deficiency is associated with *in vivo* lipid peroxidation (133). In the case of chlorinated hydrocarbons or hydrazines, various radical or electrophilic metabolites could also be responsible for initiating the carcinogenesis. The induction of peroxisomes by hypolipidemic drugs suggests that H_2O_2 could also be involved in the initiation (134). However, the only initiating species reported for the induction of liver cancer by a choline-methionine-deficient diet (in the absence of added chemical carcinogens) may be formed as a result of the associated lipid peroxidation in the nuclei (135,136).

Various studies have appeared on the modification of DNA by lipid peroxidation. DNA isolated from a vesicular gland microsome- ^{14}C -arachidonate reaction mixture was found to be irreversibly bound with ^{14}C -arachidonate oxidation products. Furthermore, DNA isolated from a peroxidizing arachidonate reaction mixture containing microsomes from lung, kidney, platelets, brain and testes was found to fluoresce with E_{excit} at 350 nm and E_{em} at 430 nm. Prostaglandin synthetase appeared to be the catalyst as indomethacin prevented this (137). The similarity of this fluorescence to lipofuscin and to DNA reacted with a malondialdehyde, a breakdown product of lipid peroxides, (138) suggests that aldehyde decomposition products may react with the amine groups of nucleic acid bases to form fluorescent Schiff bases. DNA reacted with malondialdehyde at pH 4.2 or temperatures exceeding 60°C results in a loss of template activity and decreased hyperchromicity, probably as a result of interstrand crosslinking. This has recently been demonstrated in liver and testes nuclei isolated after feeding 1,3-propanediol to rats suggesting that the crosslinking is not repairable *in vivo* (139). The crosslinking seems to involve interstrand guanine-cytosine bases in the interior of DNA rather than paired bases which could explain why they may not easily be recognized by a repair system. Enzymic digestion by the DNA, however, failed to release fluorescent nucleotide adducts. Malondialdehyde also crosslinked DNA to histones (138). However, the mutagenicity of malondialdehyde is probably due to DNA adduct formation rather than interstrand crosslinking or polymerization (140).

Incubation of linoleic acid hydroperoxide with supercoiled pBR 322 plasmid resulted in nicking of the DNA. The site of cleavage was determined by DNA sequence analysis using 3'-end and 5'-end-labeled DNA fragments as substrates. Cleavage occurred at the position of guanine nucleotide (141), presumably following a peroxyl radical attack of the purine ring. Single-strand DNA breaks can also be produced in isolated rat liver nuclei

with organic peroxy radicals (142). ESR evidence by other investigators indicates that a guanine radical is formed which is stabilized in the DNA. Polyriboguanilyc acid, but not other polyribonucleotides, gave a similar signal (143). The energy of the highest occupied molecular orbital of guanine is the highest and thus the most easily oxidized of the nucleic acid bases (144).

Oxidative Activation in Intact Cells

The polymorphonuclear leukocyte has a powerful oxygen activation mechanism used to carry out its bactericidal function. Phagocytosis or interaction of the plasma membrane with a variety of stimulatory agents (including the tumor promoter phorbol myristate acetate) results in a greatly increased cyanide respiration leading to the production of activated oxygen species. We have shown that ^{14}C -phenol, benzidine, *N*-methylaminoazobenzene, and aminofluorene incubated with leukocytes are co-oxidized and bind irreversibly to leukocyte nuclear DNA only if the leukocytes are activated (85,149,150). The binding was dependent on oxygen and was decreased by sulfhydryl inhibitors and phenolic antioxidants that inhibit the respiratory burst triggered by the phorbol myristate. Both the binding and the respiratory burst were increased by low concentrations of azide, presumably as a result of enhanced H_2O_2 levels due to intracellular catalase inhibition. However, higher azide and cyanide concentrations prevented binding without affecting the respiratory burst, indicating that myeloperoxidase is a catalyst for the binding. Granules from the activated leukocytes or myeloperoxidase also catalyzed extensive binding to calf thymus DNA with H_2O_2 and had a similar sensitivity to cyanide as the leukocytes. A 20- to 40-fold higher level of binding was found, indicating that proteins and hydrogen donors protect the nuclear DNA in the intact cell (150). A leukocyte-catalyzed activation of carcinogens may play a role in explaining the enhanced incidence of cancer associated with chronic inflammatory states. Furthermore, these cells or precursor cells found in bone marrow may play a role in benzene-induced leukemia as a result of activation of the phenol metabolite.

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Photoactivation

Photodermatological toxic reactions can often occur within a few hours of taking certain drugs. Photoallergic reactions involving an immunological mechanism is often involved resulting in eczema or bullous eruptions. These drugs include the tranquilizer phenothiazines (e.g., chlorpromazine and promazine); the antihistamine phenothiazines (e.g., promethazine); the antibiotic tetracycline, and anticancer anthracyclines. The reactive species formed that bind to macromolecules or cause membrane damage often include organic radicals or hydroxyl radicals.

Promazine photoexcitation results in the formation of cation and hydroxy radicals which cause DNA single-strand breaks but do not photobind (151). The hydroxy radical is suggested to be formed by a Haber-Weiss reaction from superoxide formed by a reaction between molecular oxygen and the photoejected electrons. Photoexcited chlorpromazine rapidly undergoes photodechlorination, yielding chlorine and promazynyl radicals which photobind to the guanine of DNA (152). Chlorpromazine complexed with DNA photobinds much less readily (152). The anthracycline antitumor agent, daunomycin, can produce photodermatological side effects including contact dermatitis and severe skin damage. Photosensitized daunomycin can result in hydroxyl radical mediated DNA strand breaks (153).

Light and acridine orange induce the formation of peroxide radicals in DNA (154). It is believed that the excited dye triplet state reacts with thymine to form anion radicals and with guanine to form cation radicals. Photosensitized guanine modification and hydroxyl radical mediated DNA strand breakage with proflavine has been reported (155). Base substituted mutations are also formed rather than the frame-shift mutations formed in the dark (156).

A mixture of hematoporphyrin derivatives, when given intravenously, is retained selectively in malignant tumor and tumor necrosis occurs when red light is then applied to the tumor (157). This method is now used in the treatment of various cutaneous and cancers of the bladder, bronchii, and gynecological organs. Guanine residues of DNA are modified by the singlet oxygen or hydroxyl radicals formed. Hydroxyl radical formation in cellular species has been detected by 5,6-dihydroxydihydrothimine formation and spin adduct formation (158).

Blood transfusion can result in the transmission of Chagas disease and the triarylmethane dye, gentian violet (crystal violet) is widely used by blood banks in attempts to eliminate this. Gentian violet is also used for the control of fungal and intestinal parasites in humans. Visible light also catalyzes the trypanocidal effects of the triarylmethane dye, gentian violet (159). Light acts by catalyzing the reductive activation by a hydrogen donor to a carbon-centered free radical as demonstrated by ESR. Genetic toxicity was also enhanced (160).

Conclusion

Free-radical metabolites can be generated metabolically by a one-electron reductase-catalyzed reduction or a "peroxidase" catalyzed oxidation or by photoactivation of a wide variety of aromatic xenobiotics. Radicals may also be generated during lipid peroxidation. Some radicals can react with DNA directly and probably result in guanine radical formation. Other radicals may intercalate DNA or bind covalently or noncovalently as a dismutation product or as a dimer, trimer or polymeric product. Modification to the DNA can result in single-strand breaks, loss of template activity, and crosslinking. The binding can prevent enzymic digestion. In some cases, the radicals react with oxygen, resulting initially in its reduction to superoxide anion radical before conversion to DNA reactive oxygen species. Most radicals probably do not interact with DNA.

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